20030206005

UNCLASSIFIED
SECURITY CLASSIFICATION OF THIS PAGE

DTIC FILE COPY

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION: Unclassified	1b. RESTRICTIVE MARKINGS
AD-A217 783	3. DISTRIBUTION/AVAILABILITY OF REPORT: Approved for Public Release; Distribution Unlimited
	5. MONITORING ORGANIZATION REPORT NUMBER(S): AFOSR-TR- 90-0086
6a. NAME OF PERFORMING ORGANIZATION: The Ohio State University Research Foundation	6b. OFFICE SYMBOL (If applicable):
6c. ADDRESS (City, State, and ZIP Code): 1314 Kinnear Road, Columbus, Ohio 43212	7a. NAME OF MONITORING ORGANIZATION: AFOSR
8a. NAME OF FUNDING/SPONSORING ORGANIZATION: Air Force Office of Scientific Research	8b. OFFICE SYMBOL (If applicable): NL
8c. ADDRESS (City, State, and ZIP Code): Building 410, Bolling Air Force Base, D.C. 20332	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: AFOSR-86-0129
	10. SOURCE OF FUNDING NUMBERS: PROGRAM ELEMENT NO. 61102F; PROJECT NO. 2312; TASK NO A5; WORK UNIT ACCESSION NO.

DTIC ELECTE FEB 08 1990 S E D

11. TITLE (Include Security Classification): Mechanisms of Chemical Modulation and Toxicity of the Immune System

12. PERSONAL AUTHOR(S): Melinda J. Tarr

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Final	FROM 4/15/86 TO 10/14/89	1989 December 15	

16. SUPPLEMENTARY NOTATION

17. COSATI CODES		
FIELD	GROUP	SUB-GROUP

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number): 1,1-Dimethylhydrazine; interleukin 1 and 2; Corynebacterium parvum-induced immunosuppression; hydrogen peroxide; L3T4, LYT-2; Ia antigen; IL2 receptor; intracellular calcium;

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

The final three and a half years of our research with 1,1-dimethylhydrazine (UDMH) have focused on delineating the mechanisms of UDMH-induced immunomodulation. Actions of UDMH which could correlate with its immunoenhancement effects include: 1.) Interference with interleukin 2 (IL2) activity by suppression of IL2 receptor expression; 2.) Non-specific stimulation of intracellular ionized calcium levels in lymphocytes; 3.) Interference with activated macrophage suppressive effects (as evidenced by reversal of Corynbacterium parvum-induced immunosuppression, as well as interference with chemiluminescence and prostaglandin E_2 production). Other mechanisms for the immunomodulatory effects of UDMH which were ruled out include: 1.) UDMH does not interfere with the production or activity of hydrogen peroxide, a "normal" endogenous immunosuppressant; 2.) UDMH does not alter the absolute or relative numbers of the T-lymphocyte subsets L3T4 (helper cells), Lyt-2 (suppressor/cytotoxic cells), or Thy1.2 (all T cells);

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT: ☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT ☐ DTIC USERS	21. ABSTRACT SECURITY CLASSIFICATION
22a. NAME OF RESPONSIBLE INDIVIDUAL: T Jan Cerveny, Lt Col, USAF	22b. TELEPHONE (Include Area Code): 202-767-5021 / 22c. OFFICE SYMBOL: NL

DD Form 1473, JUN 86 — Previous editions are obsolete. — SECURITY CLASSIFICATION OF THIS PAGE

4 JAN 1990

THE OHIO STATE UNIVERSITY

Mechanisms of Chemical Modulation and Toxicity Of the Immune System

Melinda J. Tarr
Department of Veterinary Pathology

U.S. Air Force
Air Force Office of Scientific Research
Bolling Air Force Base, D.C. 20332

Grant No. AFOSR-86-0129
Final Report

December 1989

Mechanisms of Chemical Modulation and Toxicity of the Immune System

Melinda J. Tarr

Department of Veterinary Pathology

Final Report for the Period of

April 15, 1986 - Oct. 15, 1989

U.S. Air Force

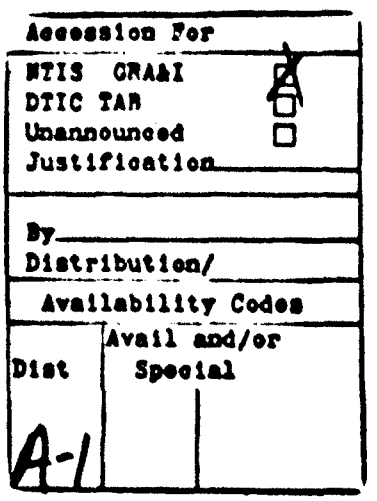
Air Force Office of Scientific Research/Pkd

Bolling AFB, D.C. 20332

Program Manager: Major T. Jan Cerveny

Grant No. AFOSR-86-0129

December 1989



I. Introduction

The work performed during the last three years represents the culmination of a project which started in 1979. The initial objectives were to identify and characterize the immunotoxic effects of various compounds of interest to the Air Force. Early in the course of the experiments, one particular compound, 1,1-dimethylhydrazine (UDMH), was found to have rather unusual properties in that it augmented many immune functions at low to moderate exposure levels, and had no effect or suppressed some immune functions at higher exposure levels. The immunoenhancement property raised concern that UDMH could potentially cause autoimmune disease; in fact, other hydrazine containing compounds are known to do so. Subsequent efforts were directed at further characterizing the immunomodulatory effects of UDMH, and delineating the mechanisms by which it exerted these effects, particularly the immunoenhancement. The following list summarizes the significant effects of UDMH that we have noted during the first 7 years of the project.

A. Effects of UDMH on immune function

1. Enhancement of Jerne plaque response (number of antibody-producing cells, in vivo exposure).
2. Enhancement of allogeneic lymphocyte response (in vivo and in vitro exposure)
3. Inhibition of concanavalin A activates suppression T-cell activity (in vivo and in vitro exposure).
4. Biphasic effect (enhancement at low concentration; suppression at higher concentration) on lipopolysaccharide-induced B-cell proliferation.
5. Suppression of concanavalin A-induced T-cell proliferation (in vitro exposure).

B. Effects of UDMH on monocyte/macrophage function.

1. Suppression of prostaglandin E_2 (PGE_2) synthesis (in vitro exposure)
2. Suppression of chemiluminescence (in vitro exposure)
3. Suppression of phagocytosis and killing (in vitro exposure)

The results of these experiments suggested that UDMH could exert its immunoenhancement effects by interference with suppressor T-cell activity and/or by suppression of PGE_2 synthesis by macrophages (PGE_2 is a natural down-regulator of the immune response). Numerous other potential mechanisms of immune modulation exist, however, and the last three years have been spent exploring some of these potential mechanisms.

II. Research Objectives:

The following is a list of objectives for the past three years which we have attained or are finishing up:

A. Characterization of the in vivo and in vitro effects of UDMH on interleukin 1 synthesis and activity.

B. Characterization of the in vivo and in vitro effects of UDMH on interleukin 2 (IL2) synthesis and binding.

C. Evaluation of the in vitro and in vivo effects of UDMH on IL2 receptor expression using flow cytometry.

D. Evaluation of the in vivo effects of UDMH on Corynebacterum parvum-induced suppression of lymphocyte blast transformation and IL2 production.

E. Evaluation of the effects of UDMH on production of superoxide anion (O_2-) by macrophages and on hydrogen-peroxide (H_2O_2)-induced suppression of lymphocyte blast transformation.

F. Determination of the in vitro and in vivo effects of UDMH on the distribution and expression of T-lymphocyte subset antigens (Thy 1.2, Lyt-2, L3T4) and immune response (Ia) antigens using flow cytometry.

G. Determination of the effects UDMH on changes in intracellular calcium during lymphocyte activation.

H. Determination of the effects of UDMH on membrane potential.

I. Determination of the effects of UDMH on the production and activity of a suppressive lymphokine, Soluble Immune Response Suppressor (SIRS).

III. Status of Research

A. Work completed before last annual report.

The rationale, methods and results of several of the listed objectives have been detailed in the last two annual reports (June 1987, May 1988). The results of those experiments will be briefly summarized, and the original or modified tables and graphs will be included in Appendix I.

1. Interleukin 1 (IL1) synthesis and activity: The effects UDMH on IL1 were investigated because IL1, produced by macrophages, is an important up-regulatory molecule in the initial phase of the immune response. The experimental results indicated that UDMH slightly suppresses the production of IL1 (Table 1) and markedly suppresses IL1 activity (Table 2). Further experiments suggest that UDMH exerts the latter effect at least partly by blocking lymphocyte IL1 receptor generation or expression (Table 3).

2. Interleukin 2 (IL2) synthesis and activity: IL2 is also an important lymphokine in the immune response, particularly in proliferation and differentiation of lymphocytes once the immune response has begun. Several experiments were done to evaluate the effects of UDMH on IL2.

   a. In vivo treatment: A slight suppression of IL2 production was seen by splenocytes from UDMH-treated mice (Table 4).

   b. In vitro exposure: Splenocytes exposed to UDMH produced slightly less IL2, and IL2 production by EL-4 cells, a cloned IL2-producing lymphocyte cell line was significantly suppressed in a dose-related fashion by UDMH (Table 5).

   c. IL2 activity: The proliferation of CTLL-20 cells, an IL2-dependent T-cell line, was suppressed in a dose related manner by UDMH (Table 6).

   d. Effects of UDMH on the IL2 molecule: Incubation of UDMH with IL2-containing supernatant did not affect the IL2 activity (Table 7).

   e. IL2 binding: Once the IL2 receptor was generated by the lymphocyte, UDMH did not affect the binding of IL2 onto the receptor (Table 8).

3. Effects of UDMH on Corynebacterium parvum (c.p.)-induced suppression of lymphocyte functions: Since c.p. is a potent macrophage activator and immunosuppressant, and other experiments indicated that UDMH interferes with activated macrophage functions, the in vivo effects of UDMH treatment on c.p.-treated mice were examined. Experiments showed that UDMH partially reversed c.p. induced suppression of IL2 production (Table 9), and lymphocyte blast transformation (Table 10), especially during the first 24 hours. It was concluded from these experiments that UDMH interferes with the immunosuppressive effects of c.p., possibly by interfering with the suppressive effects of overwhelming macrophage activation.

B. Final results and conclusions of other objectives:

For the other objectives, no or partial results were presented in previous reports. The rationale, methods, final results, and significance will be repeated and/or updated here.

1. Interleukin 2 (IL2) receptor expression.

a. This experiment was planned to be done with all the other experiments dealing with the effects of UDMH on IL2, but was delayed until the appropriate reagents were acquired. The rationale for doing the experiment was to determine if UDMH exerts its suppressive effect on IL2 activity by interfering with IL2 receptor expression. The experiment has at last been completed.

b. Method: IL2 Receptor expression in both in vivo and in vitro UDMH treated mouse splenocytes was determined by the use of monoclonal anti-IL2 receptor antibody and flow cytometry. Splenocyte cultures or CTLL-20 cells were stimulated with mitogen to obtain IL2 receptor expression. After 24 and 48 hours incubation, cells were fixed in 1% paraformaldehyde and stained by indirect immunofluorescence. Cells were assayed for receptor expression on an EPICS 753 flow cytometer.

c. Results: Experiments showed that UDMH decreased the number of cells expressing IL2 receptor at both 24 and 48 hours in vitro, however these results were not significant as determined by the student T-test and ANOVA (Table 11).

d. Conclusions from all experiments dealing with the effects of UDMH on IL1 and IL2: To summarize, these experiments suggest that UDMH suppresses IL1 activity by interfering with IL1 receptor expression on the target lymphocytes, and it interferes with IL2 activity by an uncertain mechanism, part of which may be interference with IL2 receptor expression on T-lymphocytes. It is possible that these effects are induced by a common mechanism.

These results correlate well with the previous observation that UDMH suppresses mitogen-induced lymphocyte blast transformation, as the production and activity of both IL1 and IL2 are necessary for lymphoproliferation during an immune response. However, the significance of these results in terms of the in vivo and in vitro immunoenhancement effects of UDMH is uncertain. It is difficult to extrapolate from such specific in vitro experiments as those with IL1 and IL2 to what occurs in the intact animal. One would logically conclude that the immune response would be suppressed if IL1 and IL2 activities are decreased; however, it has been shown that suppressed IL2 responses occur in animals and human patients with some autoimmune diseases, of which aberrent enhancement of certain other immune functions is a feature. Hence, these results could be consistent with the broad supposition that UDMH could cause autoimmune disease, as other hydrazine-containing compound are known to do.

2. Effects of UDMH on superoxide anion (O_2-) production by macrophages, and on hydrogen peroxide (H_2O_2)-induced suppression of lymphocyte blast transformation.

   a. Rationale: These experiments were done to further delineate the mechanism by which UDMH interfere with the immunosuppressive activity of c.p.-activated macrophage. One of the mechanisms attributed to c.p.-induced immunosuppression as well as down-regulation in a normal immune response, is H_2O_2 production by macrophages once they are activated. We wanted to determine if UDMH interferes with either the production of H_2O_2 (by measuring O_2- as the intermediate) by macrophages or the suppressive effects of H_2O_2.

   b. Methods:

      1) Lymphocyte Blast Transformation Assay - Splenocytes at 10^6cells/ml were plated in quadruplicate in 96 well microtiter plates and the mitogens Con A and LPS, or cell culture media (control) were added. UDMH at 0, 15, and 25 μg/ml and H_2O_2 at 0, 10, 20, 30 and 40 μM were then added. Cultures were incubated at 37°C in 5% CO_2-humidified incubator for 66 hours. Cell suspensions were pulsed with 10 μl (0.5μCi) tritiated thymidine for 6 hours, harvested on glass microfiber filters and counted by liquid scintillation spectrometry. Results were analyzed by the student's T-test and ANOVA.

      2) Macrophage-Superoxide Anion Assay - Thioglycollate-elicited peritoneal macrophages were placed in 96 well microtiter plates at $3X10^5$cells/well and incubated for 2 hours in 5% CO_2 at 37°C. Cell monolayers were then exposed to 100 μl of UDMH at 0, 25, 50, 100, 200, and 400 μM for 2 hours. Cell monolayers were then washed with cell culture media and incubated with 100 μl cytochrome C solution, with and without TPA to stimulate superoxide anion. Cytochrome C reduction by superoxide anion was measured by U.V. absorption spectophotometry at 550nm.

   c. Results: These experiments showed that UDMH does not interfere with either O_2-production (Table 12) or H_2O_2-induced suppression of LBT (Table 13 and 14), except at the highest, slightly toxic concentrations.

d. Significance: It was concluded from these results that UDMH does not exert its immunoenhancement effects, or its interference with activated macrophage functions, by inhibition of H_2O_2 production or activity. UDMH-induced interference with prostaglandin E_2 synthesis (reported earlier) remains as a possible mechanism.

3. Effects of UDMH on expression of T-lymphocyte antigens and immune response (Ia) antigens using flow cytometry.

a. Rationale

1) T-lymphocyte antigens: T-lymphocytes bear surface antigens which correlate with their function. Murine lymphocytes can be divided into helper/inducer cells expressing the L3T4 surface antigens and suppressor/cytotoxic cells bearing Lyt-2 surface antigens. Autoimmune or immunodeficient diseases have been characterized by alterations in the ratio or absolute number of these lymphocyte populations. Flow cytometric analysis of expression of these cell surface antigens upon exposure to UDMH both in vivo and in vitro were examined.

2) Ia antigens: The Ia antigen is a cell surface marker and product of an immune response gene. It is present on B cells and macrophages (antigen presenting cells) and suppressor T-lymphocytes. Other T-lymphocytes interact with the Ia antigen via a receptor molecule to allow optimum immune response. Alteration of or interference with Ia antigen expression may be responsible for the immunomodulatory effects of UDMH. Flow cytometric analysis of Ia antigen expression on B cells and macrophages of in vitro and in vivo UDMH exposed murine spleen cells have been examined.

b. Methods

1) Lymphocyte antigens: Lymphocytes were isolated from the spleen, mesenteric lymph nodes, and thymus of normal and UDMH-treated mice. Adherent cells were removed and nonadherent cell suspensions were incubated with UDMH (cells from normal mice only). Direct immunofluorescence staining was then performed using fluorescein and phycoerythrin-conjugated monoclonal anti-L3T4, Lyt-2, or Thy 1.2 (a general T-cell marker) antibodies. These cells were then evaluated

for percent cells expressing antigens and density of antigen per cell using flow cytometry.

2) Ia antigen: Spllen cells from normal or UDMH-treated mice were enriched for adherent populations of B-cells and macrophages. Cells were incubated with UDMH (normal cells only) and stained by indirect immunofluorescence with a monoclonal anti-Ia antibody. They were evaluated as described above using flow cytometry.

c. Results:

1) Lymphocyte antigens: The results show that UDMH did not significantly alter lymphocyte subpopulations defined by Thy1.2, L3T4 or Lyt-2 either in vivo or in vitro. However, at 75 mg/kg, the thymocyte $T_h$cell subpopulation defined the by marker L3T4 was significantly decreased from control (see Figures 1-7).

2) Ia antigen: Expression of Ia antigen on adeherent murine splenocytes was not significantly affected in mice exposed to UDMH, although a slight concentration dependent increase was noted (Table 15).

d. Significance: UDMH did not significantly affect murine lymphocyte subpopulations or expression of Ia antigen as determined by flow cytometric methods. This analysis is important in that it points out that modulation of the immune response by UDMH does not occur as a result of altered lymphocyte subpopulations or MHC Class II antigen expression. Since the lymphoid subpopulations were phenotypically normal, UDMH induced alteration of the normal immune response must occur by functional mechanisms not related to alteration of cell populations or their antigen expression.

4. Effects of UDMH on changes in intracellular calcium during lymphocyte activation.

a. Rationale: Calcium (Ca^{2+}) acts as an intracellular second messenger in many cellular events. Elevation of intracellular ionized calcium (Ca^{2+}) concentrations has been shown to occur within minutes following exposure ot lymphocytes to mitogens, and is considered to be one of the early events necessary to trigger lymphocyte activation and proliferation following exposure to mitogen or antigen. Since UDMH is a membrane permeable molecule, it is possible that UDMH could exert its

immunomodulatory effects by altering membrane Ca^{2+} flux and thus intracellular Ca^{2+} levels. The level of intracellular calcium can be easily measured with a cytoplasmically trapped fluorescent indicator that irreversibly binds Ca^{2+}. This assay is currently in use in our laboratory for other research projects.

b. Methods: Normal lymphocytes were isolated from the spleen and thymus of female Balb/C mice by asceptic techniques. A viable single cell suspension of $1.0X10^6$ cells/ml was loaded with the membrane permeable dye Fura-2 acetoxymethylester at 1.0 μM for 30 minutes and cells washed twice with cell culture medium. Intracellular free Ca^{2+} was then measured by dual wavelength excitation spectrofluorometry in cells stimulated with media, Con A, or UDMH at 10, 25, 50 and 100 μg/ml. Changes in the intracellular free Ca^{2+} were measured by the ability of Fura-2 to change its emission spectrum upon binding of Ca^{2+}. At least 5 complete experiments were performed.

c. Results: The addition of UDMH to splenocyte and thymocyte cell suspensions caused an increase in the intracellular free Ca^{2+} concentration in murine splenocytes and thymocytes similar to that induced by Con A. The results are presented in Figures 8 and 9.

d. Significance: The increase in intracellular free Ca^{2+} exhibited by murine lymphoid cells upon addition of UDMH may be important cellular phenemenon for the immunomodulatory effects exhibited previously. Because UDMH is able to cause an influx of Ca^{2+} within these cells, they may now be in an activated state of responsiveness and thus exhibit enhanced immune responsiveness. A rise in the Ca^{2+} level may also decrease the responsiveness of lymphoid cells to certain mitogens (ie. Con A) by overloading the cell with Ca^{2+}. This rise in intracellular Ca^{2+} may be due to the ability of UDMH to perturb the normal homeostatic maintenance mechanisms of either the plasma or intracellular organelle membranes.

5. Effects of UDMH on lymphocyte membrane potential

a. Rationale: Membrane potential of a cell represents the voltage difference across its plasma membrane. These voltage differences are dependent upon the distribution of both positive and negative charges thus creating an electric potential. The ability of a cell to maintain normal membrane potential is the result of Na^+-K^+ ATPase pumps in the plasma membrane that regulate ionic concentration gradients. The ability of certain chemicals to disrupt this normal cellular homeostatic mechanism

may affect the normal function of the cells. The purpose of these experiments is to examine changes induced by UDMH in the membrane potential of normal murine lymphocytes as a possible explanation for alterations in immune responsiveness observed previously with UDMH.

b. Methods: Murine lymphoid cells at 10^6cells/ml were allowed to equilibrate with the fluorescent dye 3,3'-dipentyloxacarbocyanine for 15 minutes at 37°C. Cells were then exposed to UDMH, gramicidin (depolarizes the membrane, decreases fluorescence) or valinomycin (hyperpolarizes the membrane, enhances fluorescence). Cells were then excited at 460nm and fluorescence emission measured at 510nm by fluorescence spectrometry. Five fluorescence readings were taken for each group of cells, and then analyzed by student's T-test.

c. Results: Preliminary investigations show that UDMH depolarizes the membrane of murine lymphocytes in a concentration dependent manner (Table 16).

d. Significance: UDMH causes membrane depolarization and thus alters the normal membrane potential of lymphoid cells. The ability of the cells to recover from such an event will determine the extent to which UDMH acts to alter normal cellular homeostasis. Cells which do not return to normal may not be able to function in a normal immunoregulatory manner.

6. Determination of the effects of UDMH on the production and activity of SIRS.

a. Rationale: This is a project which we proposed several years ago, and then abandoned because of the difficulty of the technique and lack of availability of necessary reagents. Recently we have overcome those problems, and we will be able to complete this objective.

Soluble immune response suppressor substance, or SIRS, is produced in an inactive state by suppressor T-cells which have been exposed to antigens or mitogens. Once produced, SIRS is oxidized by H_2O_2 (produced by macrophages) to its active form (sometimes referred to as $SIRS_{ox}$). In its oxidized state, SIRS is capable of non-specifically and reversibly inhibiting cell proliferation, possibly by interfering with the function of protein sulfhydryls which are essential for cell division. Reducing agents such as 2-mercaptoethanol and dithiothrietol inactivate $SIRS_{ox}$, thus preventing its suppression and resulting in enhancement of

the immune response. Since UDMH is a reducing agent, it is feasible that its immunoenhancing effects are at least partly due to inactivation of $SIRS_{ar}$.

b. Methods: Con A stimulated and control splenocytes were cultured for 48 hours, alone or in the presence of UDMH. Cell culture supernatant was harvested and incubated with sephadex-G50 for 1 hour at 4°C and centrifuged 3 times. Supernatants were 0.2μm filtered and concentrated 10x by lyophilization. Samples were subjected to separation by column chromatography to size proteins. Fractions were collected and pooled, concentrated by lyophilization 10x, analyzed by SDS-PAGE and subjected to western blot analysis with monoclonal anti-SIRS antibody. Fractions corresponding to the SIRS protein were then assayed by lymphocyte blast transformation (LBT) to examine the suppressive effects of the SIRS molecule on Con A and LPS stimulated lymphoblastogenesis.

c. Results: Preliminary results of the western blot analysis show that the protein has been isolated from Con A stimulated murine splenocytes. This protein induced a titrable suppression in an LBT assay. Further experiments are planned to confirm this result as well as determine whether UDMH can act to reverse the suppressive effects of $SIRS_{ar}$ or inhibit its induction.

IV. Written Publication (cumulative list)

A. Suppression of mitogen-induced blastogenesis of feline lymphocytes by in vitro incubation with carcinogenic nitrosamides. Tarr, M.J. and Olsen, R.G. Immunopharmacology 2:191-199, 1980.

B. Differential effects of hydrazine compounds on B- and T-cell immune function. Tarr, M.J. and Olsen, R.G. AGARD Conference Proceedings No. 309, Toxic Hazards in Aviation, B3-1-7, 1981.

C. In vivo and in vitro effects of 1,1-demethylhydrazine on selected immune functions. Tarr, M.J., Olsen, R.G. and Jacobs, D.L. Immunopharmacology 4:139-147, 1982.

D. Comparison in in vitro and in vivo immunotoxicology assays. Tarr, M.J., Olsen, R.G. and Jacobs, D.L. Annals N.Y. Acad. Sci. 407:469-471, 1983.

E. Species variation in susceptibility to methylnitrosourea-induced immunosuppression. Tarr, M.J. and Olsen, R.G. Env. Path. Toxicol. Oncol. 6:261-269, 1985.

F. Chemical alteration of host susceptibility to viral infection. Tarr, M.J. In: Comparative Pathobiology of Viral Diseases, Olsen, R.G., Krakowka, S. and Blakeslee, J.R., Jr., ed. pp. 47-55, 1985.

G. Enhancement of murine mixed lymphocyte response by 1,1-dimethylhydrazine: Characterization and possible mechanism. Tarr, M.J., McKown, B.J. and Olsen, R.G. Cancer Detect. Prevent. 12:573-581, 1988.

H. In vitro modulation of macrophage functions by 1,1-dimethylhydrazine. Tarr, M.J., Olsen, R.G., Bowen, B.L., and Fertel, R.H. Toxicology in Vitro 2:215-219, 1988.

I. Effects of 1,1-dimethylhydrazine on lymphoproliferative and interleukin 2 regulatory function. In press. Environ. Contam. Toxicol., 1989.

J. Effects of 1,1-dimethylhydrazine on immunosuppression in mice treated with Corynebacterium parvum. Bauer, R.M., Frazier, D.E., Tarr, M.J., and Olsen, R.G. In preparation.

K. Effects of 1,1-dimethylhydrazine on macrophage oxygen metabolite production and immunosuppressive activity. Frazier, D.E., Tarr, M.J., and Olsen, R.G. In preparation.

L. Effects of 1,1-dimethylhydrazine on interleukin 1 production and activity. Bauer, R.M., Tarr, M.J., and Olsen, R.G. In preparation.

M. Effects of 1,1-dimethylhydrazine on lymphocyte antigen expression. Frazier, D.E., Huff, L., Tarr, M.J., and Olsen, R.G. In preparation.

N. Effects of 1,1-dimethylhydrazine on intracellular Ca^{2+} levels and membrane polarity of lymphocytes. Frazier, D.E., Wright, K., Tarr, M.J., and Olsen, R.G. In preparation.

O. Effects of 1,1-dimethylhydrazine on production and activity of soluble immune response suppressor (SIRS). In preparation.

V. Professional Personnel Associated with Research Effort

A. Melinda J. Tarr, D.V.M., Ph.D., Principal Investigator, Department of Veterinary Pathobiology, The Ohio State University, Columbus, Ohio 43210

B. Richard G. Olsen, Co-Principal Investigator, Departments of Veterinary Pathobiology, Microbiology (College of Biological Sciences), and Comprehensive Cancer Center, The Ohio State University, Columbus, Ohio 43210.

C. Donald E. Frazier, Jr., B.S., M.S. (graduate research associate)

Mr. Frazier earned his Master of Science degree in summer 1987, and is now working towards a Ph.D. He was fully supported by this grant.

D. Kurt Wright, B.S., M.S. (graduate research associate, research assistant).

Mr. Wright helped with the calcium studies for this project. He is now enrolled in medical school at The Ohio State University.

E. Laura Huff, B.S., M.S. (research associate)

As chief operator of the flow cytometer, Ms. Huff participated in studies involving the flow cytometric evaluation of lymphocyte antigens.

VI. Oral Presentations - April 1986 to Oct. 1989

A. The following abstracts were presented at the 16th Conference on Toxicology, Dayton, Ohio, October 1986:

1. Effect of unsymmetrical dimethylhydrazine on concanvalin A- and prostaglandin-induced T-cell suppressor factor-induced suppressor cell activity. Pillay, D.J., Tarr, M.J., and Olsen, R.G.

2. In vitro effects of 1,1-dimethylhydrazine on hydrogen peroxide-induced suppression of lymphocyte blast transformation. Frazier, D., Tarr, M.J., and Olsen, R.G.

3. 1,1-Dimethylhydrazine modifies the production of and response to interleukin 1 and interleukin 2. Bauer, R.M., Tarr, M.J., and Olsen, R.G.

4. Effects of 1,1-dimethylhydrazine on Corynebacterium parvum-induced immunosuppression. Tarr, M.J., Bauer, R.M., and Olsen, R.G.

B. Possible mechanisms of immunomodulation by 1,1-dimethylhydrazine. Tarr, M.J., Olsen, R.G., Bauer, R.M., and Frazier, D.E. Presented at 2nd International Symposium on Immunobiology of Clinical Oncology and Immune Dysfunctions. Nice, France, April 1987.

C. Mechanisms of immune alterations induced by xenobiotics. Tarr, M.J. Seminar presented at Institute Nazionale per la Ricerca sul Cancro, Genoa, Italy, April 1987.

D. Effect of unsymmetrical 1,1-dimethylhydrazine on interleukin 2 immunoregulatory function. Bauer, R.M., Tarr, M.J., and Olsen, R.G. Presented at the 17th Conference of Toxicology, Dayton, Ohio, November 1987.

E. Immunomodulatory effects of hydrazine compounds. Tarr, M.J., Frazier, D.E., and Olsen, R.G. Presented at 3rd Inter-American Society of Chemotherapy Conference, Clearwater Beach, Florida, January 1988.

F. Frazier, D.E., Tarr, M.J., and Olsen, R.G. The effects of 1,1-dimethylhydrazine on cell membrane potential and intracellular calcium mobilization of murine lymphoid cells. Presented at 18th Conference on Toxicology, Fairborn, Ohio November 1988.

APPENDIX

Table 1. Effects of 1,1-Dimethylhydrazine on Interleukin 1 Induction and Production by $P388_{D1}$ Cells.[a]

[UDMH] (μg/ml)	Induction (T=0)[b]	Production (T=5)[c]
0 (control)	41538 ± 7318 (s.d.)	40961 ± 7865[d]
10	46425 ± 2209	23012 ± 2423[d]
25	45857 ± 6659	19264 ± 2722[d]
50	47219 ± 3184	24546 ± 2137[d]
75	35789 ± 5598	25920 ± 2665[d]
100	50703 ± 1997	33212 ± 2308

[a]IL1 production measured using C3H/HeJ thymocyte proliferation assay. Results expressed in counts per minute (reflecting tritiated thymidine uptake by thymocytes).

[b]UDMH present during the 5 hour induction phase with phorbol myristic acetate, then removed by washing.

[c]UDMH present during the 24 hr IL1 production phase.

[d]Significantly less than control at $p \leq .025$ (Student "t" test).

Table 2. Effects of 1,1-Dimethylhydrazine on Interleukin 1 Activity.[a]

[UDMH] (μg/ml)	24 hrs	48 hrs	72 hrs
0 (control)	74857 ± 3927 (s.d.)	71555 ± 5769	61404 ± 2544
10	25023 ± 15051[b]	18042 ± 15656[b]	37307 ± 3365[b]
25	25113 ± 4837[b]	16793 ± 3955[b]	28769 ± 4825[b]
50	4937 ± 1218[b]	4080 ± 830[b]	24842 ± 4053[b]
75	4854 ± 1168[b]	1153 ± 627[b]	6928 ± 1800[b]
100	7778 ± 1254[b]	2372 ± 1487[b]	3134 ± 591[b]

[a]UDMH was added with IL1-containing supernatant (5%) and PHA to C3H/HeJ thymocytes, which were harvested 24, 48 or 72 hours later and evaluated for tritiated thymidine uptake. Results expressed in counts per minute.

[b]All treated groups were significantly different than their respective control groups at $p \leq .001$ (Student "t" test).

Table 3. Effect of UDMH on IL1 receptor generation.

Concentration UDMH (μg/ml)	Incorporation of ^{3}H-TdR (cpm)	p
0	21619 ± 2049	-
10	89266 ± 1237	<.001
25	90265 ± 1704	<.001
50	93210 ± 1704	<.001
75	89542 ± 1626	<.001
100	91742 ± 2053	<.001

Results represent the mean ± S.D. of 4 individual experiments, each of which was assayed in triplicate in the IL1 co-mitogen assay.

UDMH was added to PHA-plus IL1-stimulated thymocytes at culture initiation and the cell-free supernatant was tested for IL1 activity following 24 hours of culture.

Table 4. Effect of UDMH Administration in vivo on Lectin-stimulated IL2 Production.[a]

Dose UDMH (mg/kg)	N[b]	IL2 Activity[c] (X cpm ± S.D.)	P
0	6	25220 ± 4854	-
10	4	24753 ± 6233	.81
25	6	23245 ± 6231	.29
50	6	21886 ± 3483	.023
75	5	23558 ± 8952	.5
100	1	14404 ± 312	.001

[a]Mice were treated with and without UDMH for 7 days and splenocytes were prepared, stimulated with Con A and IL2 activity determined as described.

[b]N = number of animals

[c]X cpm 1 S.D. ^{3}H-Tdr incorporation determined from triplicate assay of N animals.

Table 5. Effect of UDMH on IL-2 Production.[a]

Cell Culture	Concentration UDMH (μg/ml)	CPM ± S.E.M.	%Inhibition[b]
EL-4	0	58787 ± 362	--
	10	44161 ± 185[c]	24.88
	25	44430 ± 552[d]	24.43
	50	40971 ± 625[d]	30.31
Splenocyte	0	28618 ± 421	--
	10	28407 ± 1351	0.74
	25	24951 ± 705	12.82
	50	26162 ± 1626	8.59

[a]Interleukin 2 was produced as described and quantified in the IL-2-dependent proliferation assay using CTLL-20 cells

[b]Percent inhibition determined by:

$$100 - \left[\left(\frac{\text{cpm of UDMH-treated cultures}}{\text{cpm of control cultures}} \right) \times 100 \right]$$

[c]Significantly less than untreated control at $P \leq 0.019$

[d]Significantly less than untreated control at $P \leq 0.001$

Table 6. Effect of UDMH on IL-2-dependent proliferation of CTLL-20 cells.[a]

Concentration UDMH (μg/ml)	CPM ± S.E.M.	% Inhibition[b]
0	76322 ± 1972	--
10	67705 ± 1745	11.3
25	65481 ± 1339[c]	14.2
50	44603 ± 4022[d]	41.6

[a]CTLL-20 cells were exposed to UDMH at culture initiation in the presence of IL-2 and the proliferative response determined as described in the text.

[b]Percent inhibition determined by:

$$100 - \left[\left(\frac{\text{cpm of UDMH-treated cultures}}{\text{cpm of control cultures}} \right) \times 100 \right]$$

[c]Significantly less than control (0 μg/ml) at $P \leq 0.02$

[d]Significantly less than control (0 μg/ml) at $P \leq 0.001$

Table 7. Effect of 1,1-Dimethylhydrazine on Activity of the Interleukin 2 Molecule.[a]

[UDMH] (μg/ml)	Proliferative Response of CTLL-20 Cells		
	4°, 24 hrs	22°, 24 hrs	37°, 24 hrs
0	106321 ± 2200 (s.e.m.)	110134 ± 2422	109335 ± 2121
50	107143 ± 2721	103714 ± 1910	101943 ± 3417
100	102491 ± 3746	104439 ± 3178	110431 ± 2031

[a]UDMH was added to IL2-containing supernatant from EL-4 cells and incubated at the indicated temperature for 24 hrs. The IL2 activity was then evaluated using the CTLL-20 proliferation assay. Results are expressed as counts per minute (reflecting tritiated thymidine uptake by CTLL-20 cells).

Table 8. Effect of UDMH on Binding of IL2 to the IL2 Receptor.[a]

Concentration UDMH (μg/ml)	IL2 Activity[b]
0	9395 ± 2609
10	9015 ± 1466
25	9208 ± 1954
50	9433 ± 2597
75	8862 ± 2123
100	10681 ± 3262

[a]CTLL-20 cells were stimulated with IL2 in the presence and absence of UDMH. Following 24 hours of incubation, IL2 remaining in the culture medium was determined in the IL2 microassay.

[b]Mean cpm ± S.D. for 3 individual experiments.

Table 9. Effects of UDMH Treatment on Interleukin 2 Production by Splenocytes from Corynebacterium parvum-treated Mice.[a]

Treatment	Counts per minute	
	24 hours	48 hours
Control (no treatment)	59714 ± 600 (s.e.m.)[b]	57410 ± 750
C. parvum	9065 ± 1000[c]	7423 ± 1150[c]
C. parvum + 25 mg/kg UDMH	12346 ± 1400[c]	20105 ± 3050[c,d]
C. parvum + 50 mg/kg UDMH	22945 ± 7100[c]	13563 ± 1550[c,d]
C. parvum + 100 mg/kg UDMH	16682 ± 2600[c]	12297 ± 950[c,d]

[a]Interleukin 2 activity determined by proliferative response of CTLL-20 cells when exposed to supernatants of splenocytes incubated with concanvalin A for either 24 or 48 hours.

[b]s.e.m. = standard error of the mean.

[c]Significantly less than control at $p < 0.04$.

[d]Significantly greater than C. parvum alone at $p < 0.04$.

Table 10. Effects of UDMH Treatment on Con A-induced Lymphocyte Blast Transformation Response of Splenocytes from Corynebacterium parvum-treated mice.[a]

Treatment	Counts per minute 24 hours	48 hours
Control (no treatment)	17193 ± 2050 (s.e.m.)[b]	93165 ± 6400
C. parvum	1931 ± 640[c]	2735 ± 500[c]
C. parvum + 25 mg/kg UDMH	13621 ± 1250[d]	15202 ± 8650[e]
C. parvum + 50 mg/kg UDMH	13028 ± 2050[d]	14749 ± 5100[e]
C. parvum + 100 mg/kg UDMH	13629 ± 8150[d]	18933 ± 5500[e]

[a]Splenocytes were cultured with Con A and evaluated for Tdr uptake after 24 and 48 hours.

[b]s.e.m. = standard error of the mean.

[c]Significantly less than control at $p < .001$.

[d]Significantly greater than C. parvum alone at $p < 0.001$; not significantly different from control.

[e]Significantly greater than C. parvum alone at $p < 0.001$; significantly less than control at $p < 0.001$.

Table 11. Effects of UDMH on IL2 Receptor Expression in Murine Splenocytes

Treatment	Percent of Cells Expressing IL2 Receptor	
	24 hours	48 hours
Control (no treatment)	4.0 ± 5.6	5.9 ± 3.9
Con A + 0 μg/ml UDMH	26.6 ± 8.6[a]	37.4 ± 10.4[a]
Con A + 10μg/ml UDMH	27.3 ± 10.4[a,b]	38.2 ± 5.2[a,b]
Con A + 25μg/ml UDMH	31.7 ± 8.9[a,b]	34.3 ± 7.2[a,b]
Con A + 50μg/ml UDMH	23.1 ± 8.7[a,b]	31.5 ± 5.9[a,b]
Con A + 100μg/ml UDMH	22.9 ± 6.5[a,b]	30.6 ± 5.5[a,b]

[a] P = 0.0004, means are significantly different from control (ANOVA)

[b] P = 0.498, means are not signifcantly different from Con A control (ANOVA)

Table 12. Effects of 1,1-Dimethylhydrazine on O_2^- Production by Elicited Macrophages.

UDMH (μM)	nanomoles O_2^-/ mg cell protein	nanomoles O_2^-/ well
0 (control)	370 ± 283	1.01 ± 0.18
25	392 ± 211	1.42 ± 0.41
50	692 ± 673	1.43 ± 0.50
100	593 ± 573	1.42 ± 0.96
200	465 ± 268	1.31 ± 0.19
400	404 ± 75	0.32 ± 1.37[a]

[a]Significantly different from control at $p \leq 0.05$ (student "t" test).

Table 13. Effects of 1,1-Dimethylhydrazine and Hydrogen Peroxide on Concanavalin A-induced Lymphocyte Blast Transformation.

H_2O_2 (μM)	0μg/ml UDMH[a] (control)	15μg/ml UDMH	25μg/ml UDMH
0	71880 ± 16525	63086 ± 13285	57224 ± 12054
10	69021 ± 12963	63927 ± 9131	56110 ± 7754
20	75599 ± 17087	68910 ± 14383	56992 ± 16514
30	62492 ± 22207	48930 ± 17524	44697 ± 9821
40	36981 ± 14392[b]	12471 ± 3069[b,c]	7118 ± 1685[b,c]

[a]Results reported in mean CPM ± S.D.

[b]$P \leq .009$ (compared to no H_2O_2 and no UDMH) (student "t" test).

[c]$P \leq .037$ (compared to no UDMH and 40 μM H_2O_2).

Table 14. Effects of 1,1-Dimethylhydrazine and Hydrogen Peroxide on Lipopolysaccharide-induced Lymphocyte Blast Transformation.

H_2O_2 (μM)	0 μg/ml UDMH[a] (control)	15 μg/ml UDMH	25 μg/ml UDMH
0	45207 ± 8389	48895 ± 16944	45707 ± 11961
10	31084 ± 12896[b]	43853 ± 17865	35072 ± 13983
20	29182 ± 10115[b]	28869 ± 12282	28365 ± 6600[b]
30	11578 ± 6135[b]	8450 ± 3028[b]	8572 ± 3547[b]
40	5663 ± 2117[b]	3132 ± 821	3122 ± 1294[b]

[a]Results are reported as mean CPM ± S.D.

[b]$P \leq .05$ (compared to no H_2O_2 at the same UDMH concentration) (student "t" test).

Table 15. Expression of Ia Antigen* on Adherent Murine Splenocytes with Exposure to UDMH In Vivo.

UDMH mg/kg	*Percent Positive Cells
0	65.3 ± 12.5[a] (4)[b]
10	69.9 ± 8.0 (4)
25	70.0 ± 8.3 (4)
50	70.0 ± 12.2 (4)
75	71.3 ± 12.7 (2)

[a]P > 0.05 means not significantly different (student's "t" test).

[b]Number of experiments.

Table 16. Effect of UDMH on Membrane Potential of Murine Lymphocytes

Treatment	Membrane Potential
Control (no treatment)	178.6 ± 2.4
Valinomycin[c]	187.2 ± 2.7[a]
10 μg/ml UDMH	168.6 ± 1.8[b]
25 μg/ml UDMH	150.8 ± 1.1[b]
50 μg/ml UDMH	123.6 ± 3.1[b]
100 μg/ml UDMH	115.5 ± 1.4[b]

[a] $P = 0.0007$, means are significantly different from control as determined by student's T-test

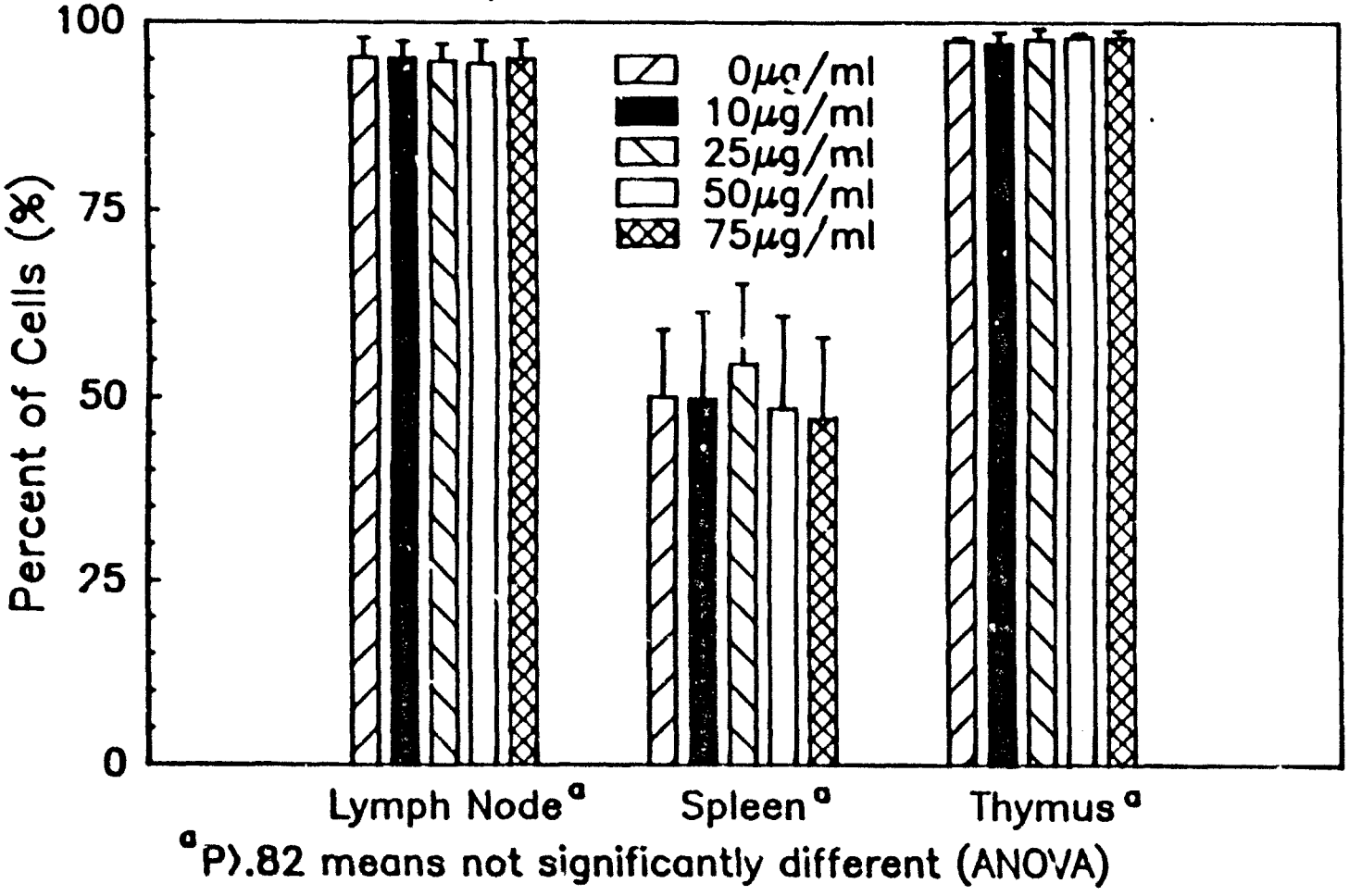
[b] $P < 10^{-5}$, means are significantly different as determined by student's T-test

[c] Valinomycin causes membrane hyperpolarization

Abstract

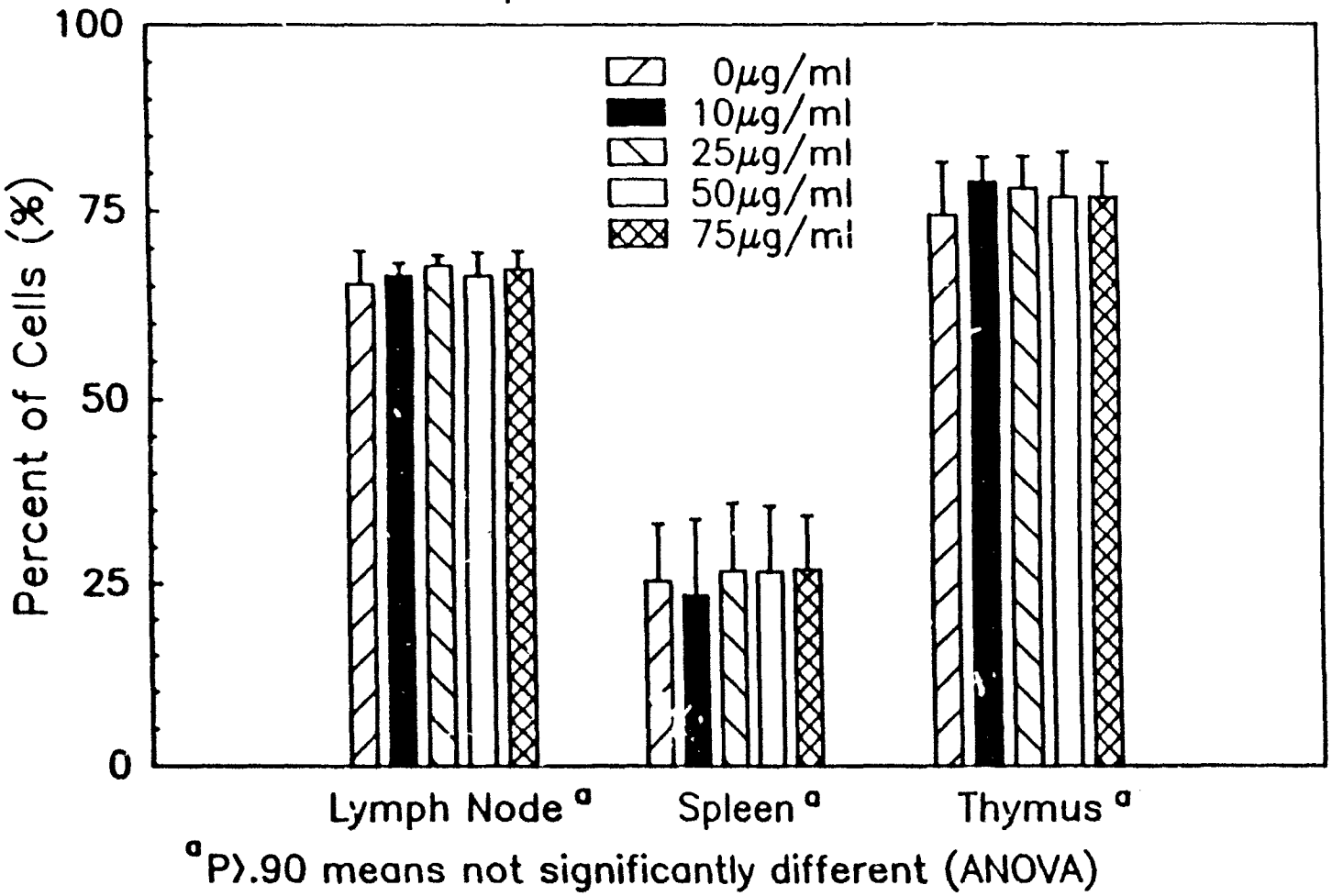
The final three and a half years of our research with 1,1-dimethylhydrazine (UDMH) have focused on delineating the mechanisms of UDMH-induced immunomodulation. Actions of UDMH which could correlate with its immunoenhancement effects include: 1.) Interference with interleukin 2 (IL2) activity by supression of IL2 receptor expression; 2.) Non-specific stimulation of intracellular ionized calcium levels in lymphocytes; 3.) Interference with activated macrophage suppressive effects (as evidenced by reversal of Corynebacterium parvum-induced immunosuppression, as well as interference with chemiluminescence and prostaglandin E_2 production). Other mechanisms for the immunomodualtory effects of UDMH which were ruled out include: 1.) UDMH does not interfere with the production or activity of hydrogen peroxide, a "normal" endogenous immunosuppressant; 2.) UDMH does not alter the absolute or relative numbers of the T-lymphocyte subsets L3T4 (helper cells), Lyt-2 (suppressor/cytotoxic cells), or Thy1.2 (all T cells); 3.) Ia antigen (immune response antigen) expression is also not affected by UDMH. One other effect of UDMH is suppression of interleukin 1 (IL1) activity by interfering with IL1 receptor expression. We are still investigating the effects on lymphocyte membrane potential and on the production and activity of Soluble Immune Response Suppressor (SIRS).

Figure 1. Effect of UDMH on Thy1.2 Antigen Expression In Vitro



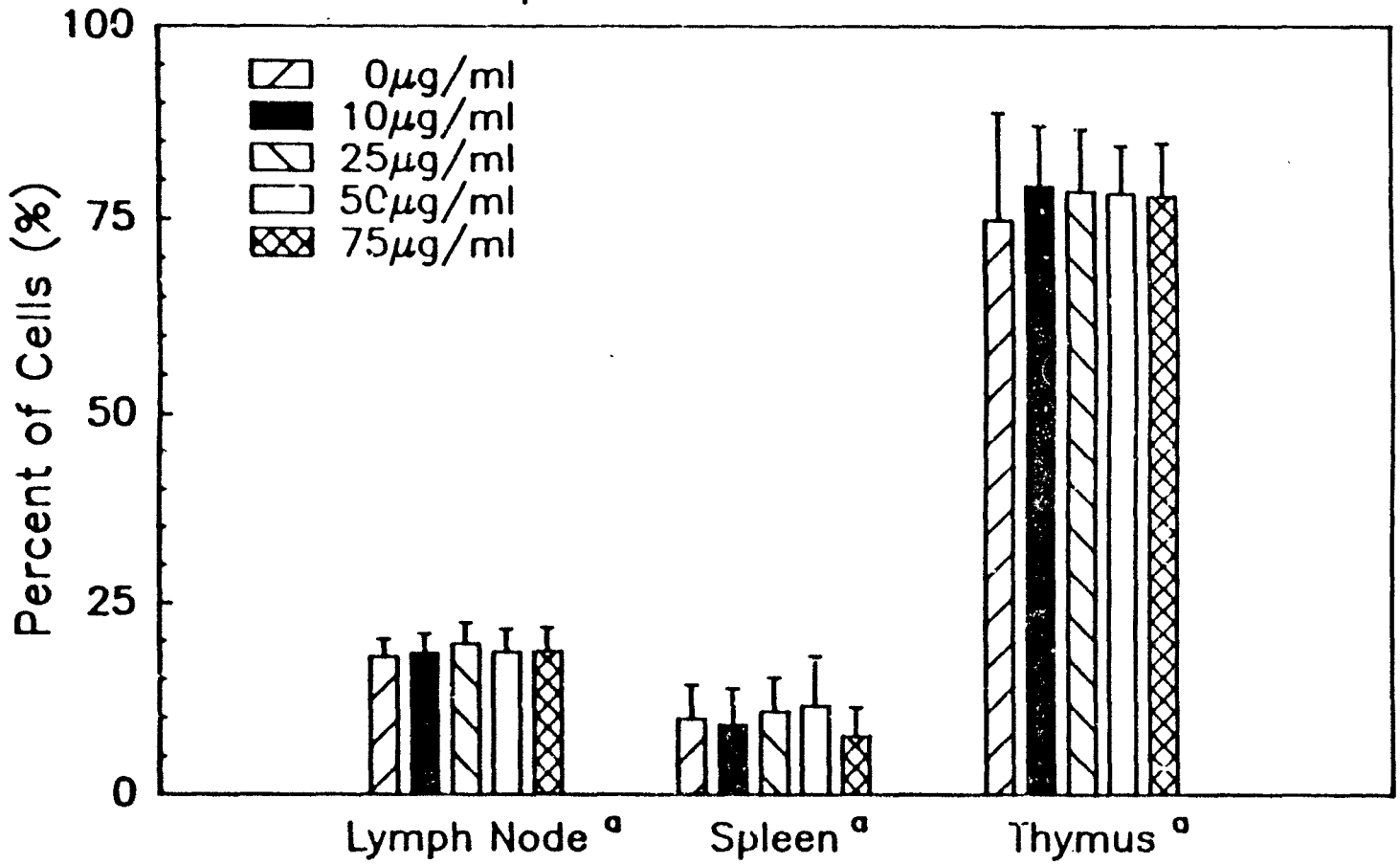
[a] P>.82 means not significantly different (ANOVA)

Figure 2. Effect of UDMH on L3T4 Antigen Expression In Vitro



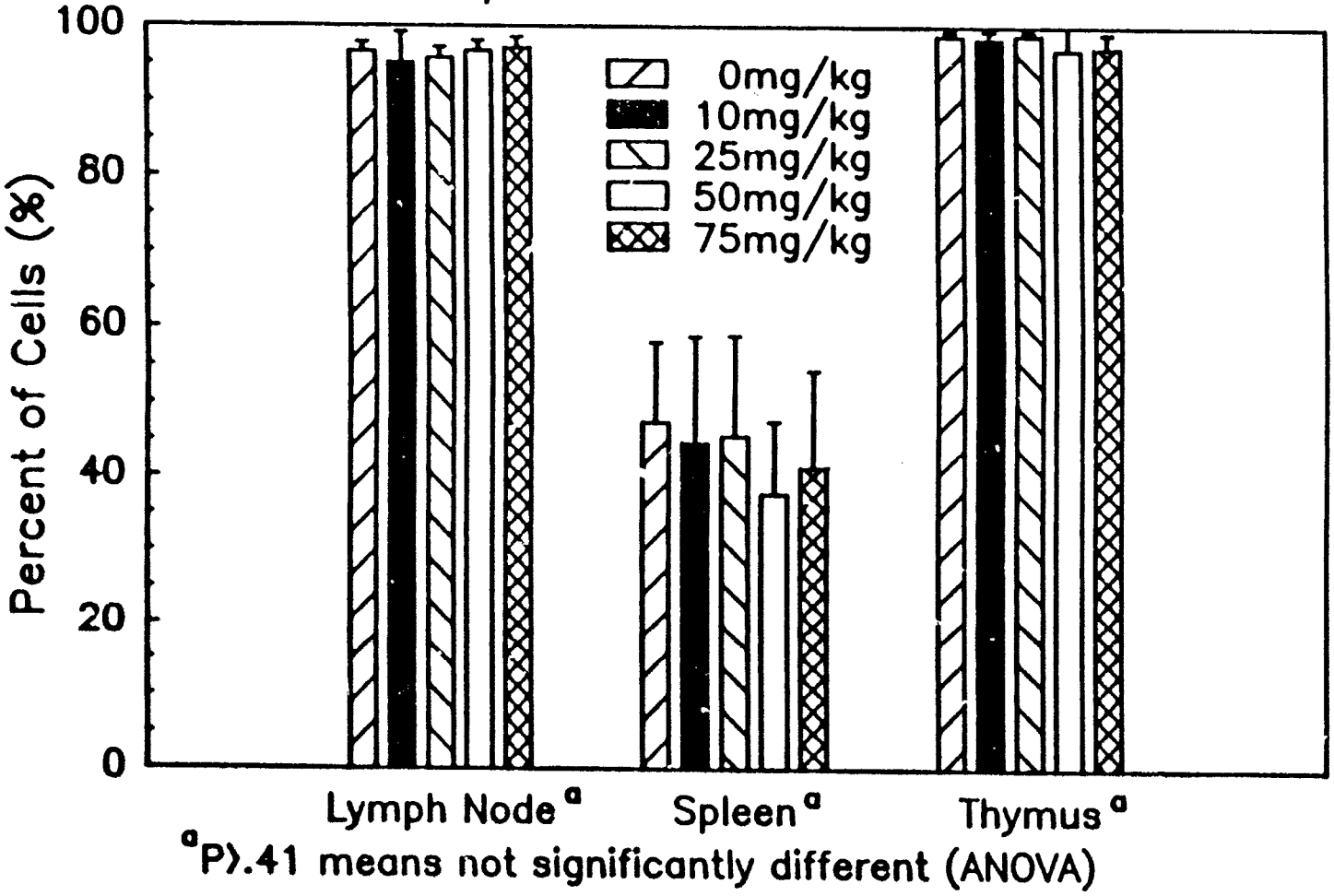
[a]P>.90 means not significantly different (ANOVA)

Figure 3. Effect of UDMH on Lyt−2 Antigen Expression In Vitro



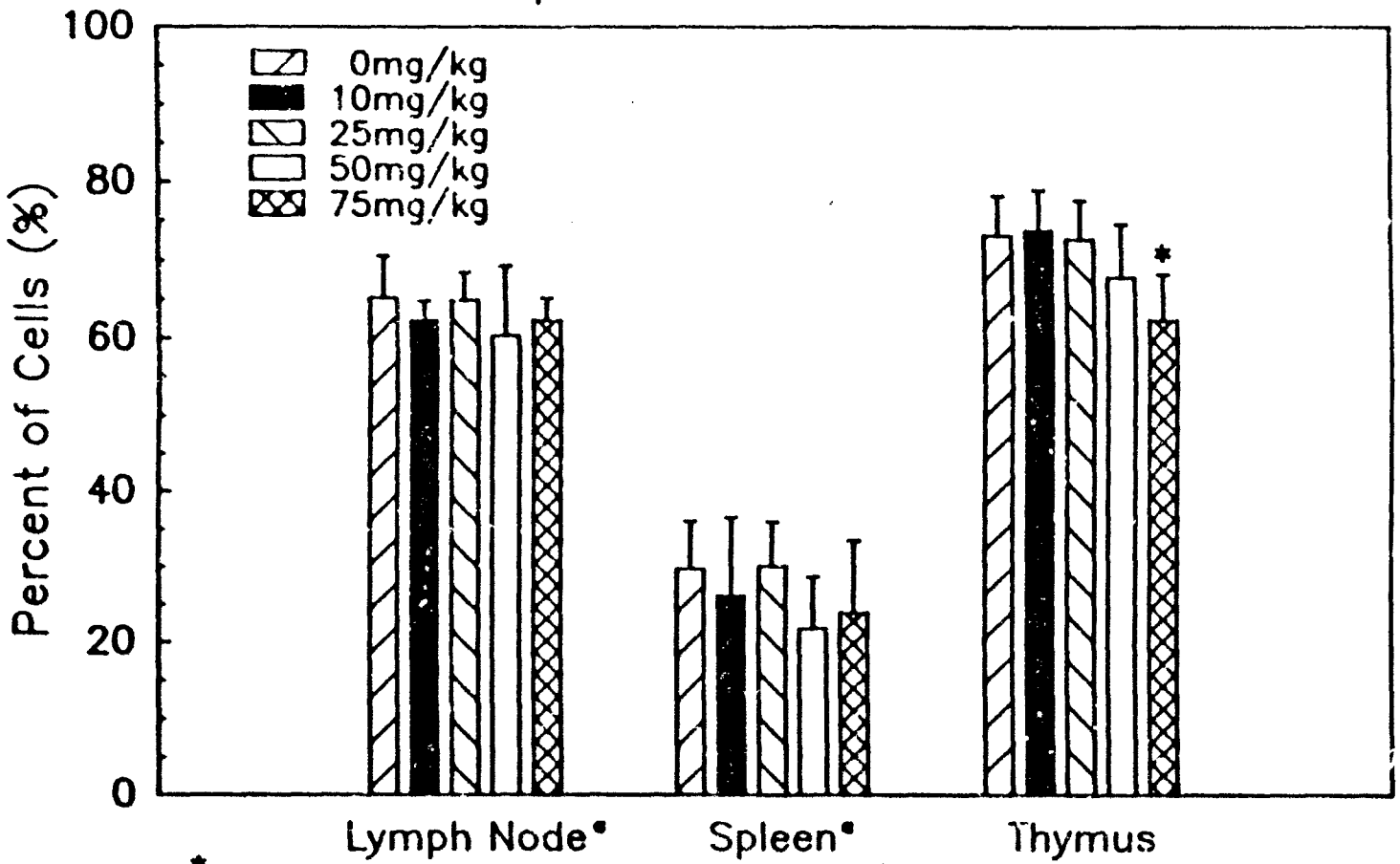
[a]P>.80 means not significantly different (ANOVA)

Figure 4. Effect of UDMH on Thy1.2 Antigen Expression In Vivo



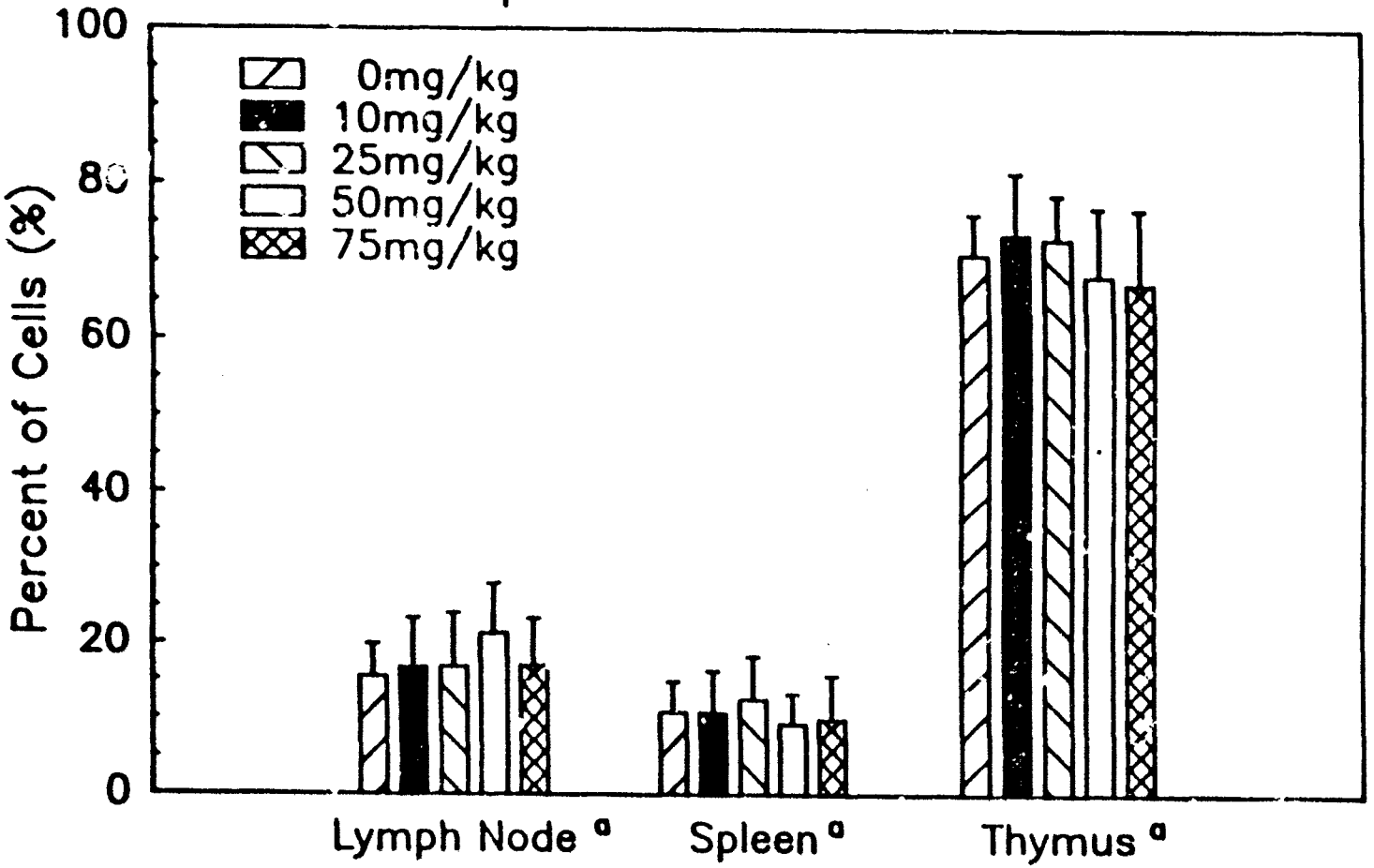
[a] P>.41 means not significantly different (ANOVA)

Figure 5. Effect of UDMH on L3T4 Antigen Expression In Vivo



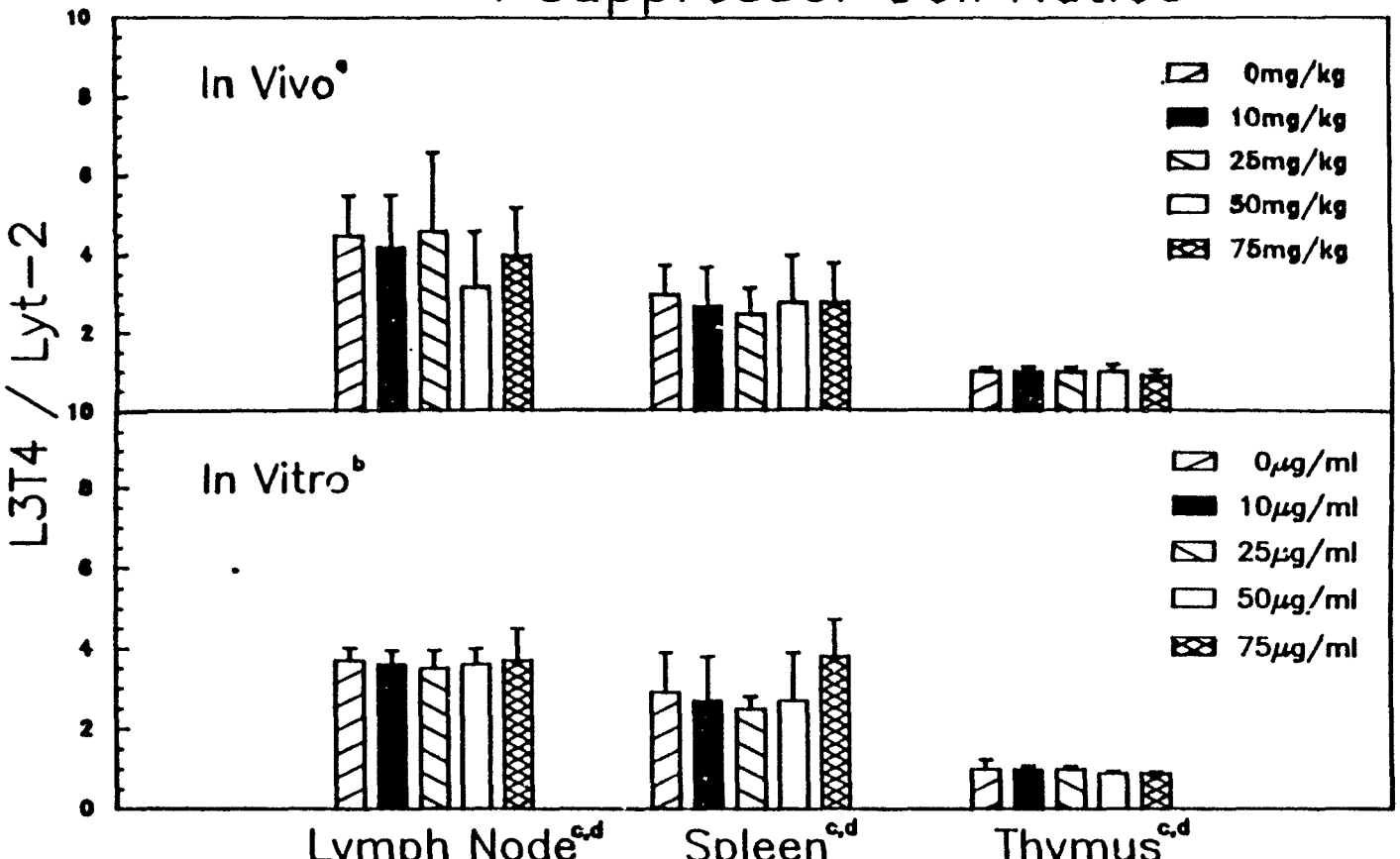
*P=.008 mean is significantly different (ANOVA)
*P>.31 means not significantly different (ANOVA)

Figure 6. Effect of UDMH on Lyt−2 Antigen Expression In Vivo



[a]P>.62 means not significantly different (ANOVA)

Figure 7. Effect of UDMH on T helper / T suppressor Cell Ratios



[a,c]P>.38 means not significantly different (ANOVA)

[b,d]P>.30 means not significantly different (ANOVA)

Figure 8. Intracellular Free Calcium in Murine Splenocytes upon Exposure to UDMH In Vitro

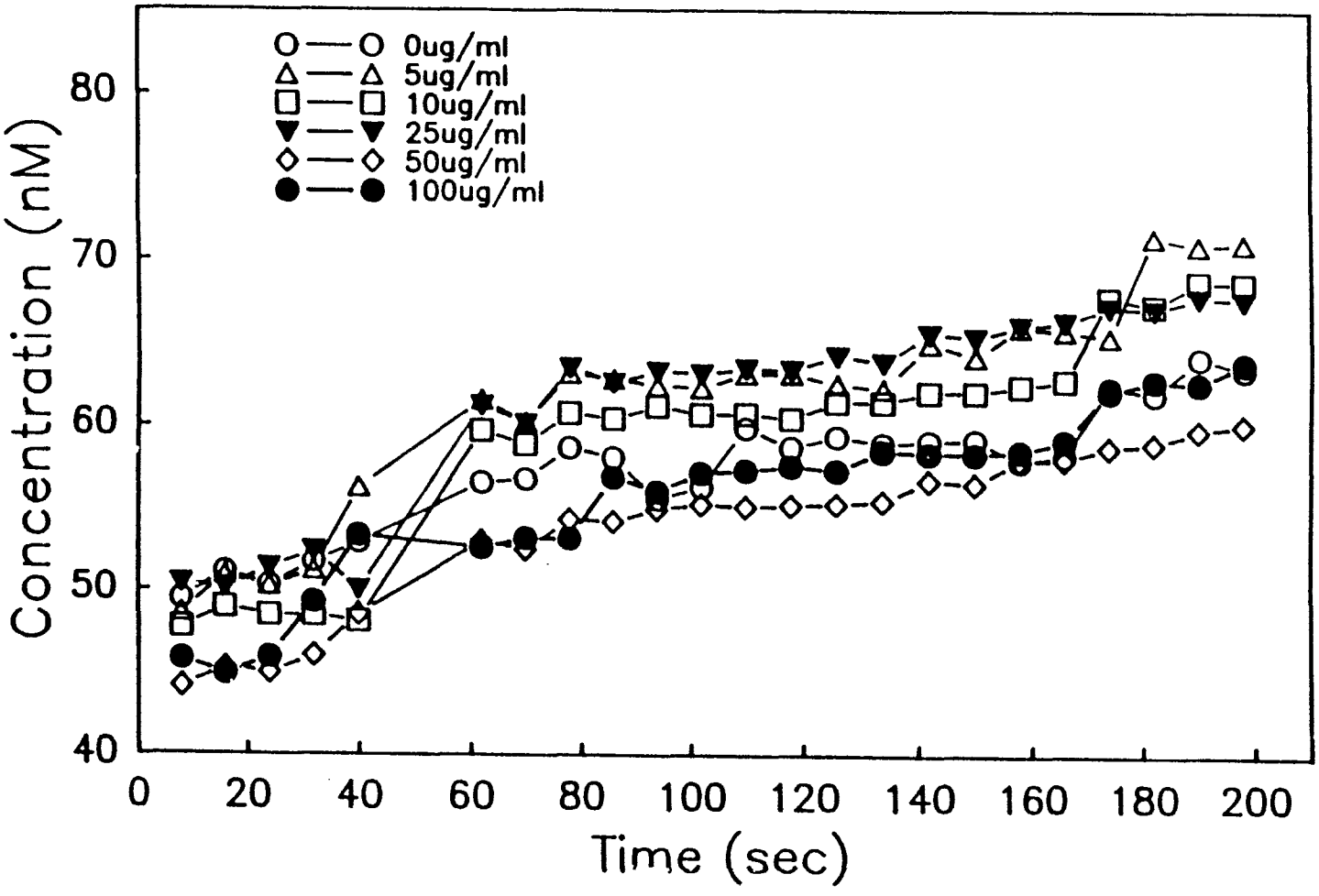


Figure 9. Intracellular Free Calcium in Thymocytes upon Exposure to UDMH In Vitro